Four-Color Flow Cytometric Detection of Retrovirally Expressed Red, Yellow, Green, and Cyan Fluorescent Proteins

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We have optimized a method for differentiating four different fluorescent proteins expressed in living cells using dual-laser flow cytometry. With this method, we provide an efficient and reliable way for simultaneously detecting and sorting multiple subpopulations of viable genetically modified cells. We have also demonstrated the development of stable, permanent cell lines expressing DsRed1, EYFP, EGFP, or ECFP.

Since its isolation from the luminescent jellyfish *Aequorea victoria*, green fluorescent protein (GFP) has become a powerful tool for researchers studying mammalian and microbiological systems. However, spectral properties inherent in the wild-type protein have limited its use in flow cytometry. EGFP (enhanced green fluorescent protein), a red-shifted GFP mutant developed by BD Biosciences Clontech overcame this limitation with an increased extinction coefficient and quantum yield when excited at 488 nm, which made it ideal for flow cytometric applications using argon-ion lasers (1).

BD Biosciences Clontech developed two additional GFP variants, enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP), which provide a broad spectrum for analyses, but still have significant overlap in their spectra. Until recently, researchers requiring the use of multicolor fluorescence for flow cytometric work were hindered from multilabeling experiments by the spectral overlap of these GFP variants and the limitations of available equipment. The recent isolation of a red fluorescent protein from the nonbioluminescent reef coral *Discosoma sp*. has expanded this range by more than 50 nm. Additionally, introduction of a human codon-optimized version, DsRed1, extends the utility of this protein even further by allowing expression in mammalian systems.

In this report, we present an optimized technique for identifying mammalian cell populations stably expressing DsRed1, EYFP, EGFP, or ECFP using dual-laser line excitation at 458 and 568 nm. We also describe an alternative three-color detection protocol for use on instruments that have only single-laser line excitation at 488 nm. These methods provide a framework for studies requiring simultaneous analysis of multiple fluorescent proteins within living cells (2, 3).

Development of stable cell lines

We cloned the genes from the plasmids pDsRed1-1, pEYFP-N1, pEGFP-1, and pECFP encoding DsRed1, EYFP, EGFP, and ECFP, respectively, into murine stem cell virus (MSCV) expression vector backbones (Figure 1). Then, we generated stable cell lines expressing these proteins by transduction of GP+E-86 ecotropic packaging cells, a derivative of the murine embryonic fibroblast cell line NIH 3T3

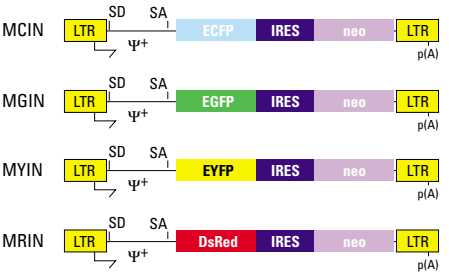

Figure 1. Retroviral vectors for expression of DsRed1, EYFP, EGFP, and ECFP transgenes. The MRIN, MYIN, MGIN, and MCIN retroviral vectors contain the DsRed1, EYFP, EGFP, and ECFP genes, respectively, under the control of the MSCV long terminal repeat (LTR) on a bicistronic transcript that also contains a downstream neomycin resistance (neo) gene linked via an encephalomyocarditis virus internal ribosome entry site (IRES). SD = splice donor. SA = splice acceptor. Ψ^+= extended packaging signal. p(A) = polyadenylation site. Adapted and reproduced in part by permission of AlphaMed Press from Hawley *et al.* (3).

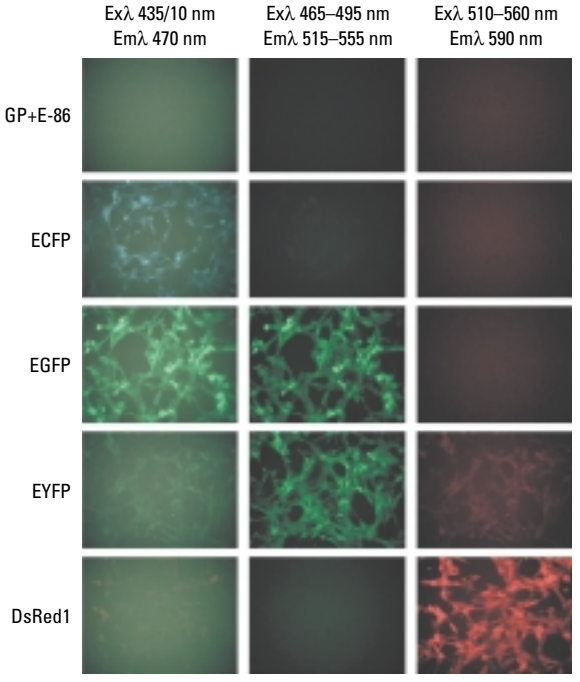

Figure 2. Fluorescence microscopy analysis of each fluorescent protein using three standard filter sets. GP+E-86 cells individually expressing DsRed1, EYFP, EGFP, and ECFP were imaged using each filter set indicated. The BV-1A filter set (EX 435/10, DM 455, BA 470) used to image the ECFP signal also captured EGFP fluorescence and, to a lesser extent, EYFP fluorescence. The B-2E/C filter set (EX 465–495, DM 505, BA 515–555) used for fluorescein isothiocyanate-like emission detected the EGFP signal and the EYFP signal. The G-2A filter set (EX 510-560, DM 575, BA 590) used for phycoerythrin-like emission detected the DsRed1 signal as well as the EYFP signal. To demonstrate spectral overlap, images were overexposed under a Nikon Eclipse TE 300 inverted fluorescence microscope equipped with a 100W mercury arc lamp and a Sony DKC-500 Digital Camera. Reproduced from Hawley *et al.* (2) by permission of Eaton Publishing.

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Four-Color Flow Cytometric Detection of Retrovirally Expressed Red, Yellow, Green, and Cyan Fluorescent Proteins...*continued*

(4), followed by G418 selection and/or cell sorting as described by Cheng *et al.* (5). Each permanent cell line demonstrated no toxicity as a result of protein expression.

Spectral overlap of fluorescent proteins

Prior to flow cytometric analysis, we evaluated the stable cell lines expressing each protein using fluorescence microscopy. We used standard filter sets that closely matched the excitation and emission maxima for each fluorescent protein. While all of the fluorescent proteins were easily seen, spectral overlap was prominent, especially among the GFP variants (Figure 2).

Flow cytometry

Next, we analyzed the cells using a FACSVantage™ SE cell sorter equipped with Coherent I-90 argon-ion and Coherent I-302C krypton-ion lasers. The primary laser was tuned to 458 nm for detection of the three GFP variants and the second laser was tuned to 568 nm for detection of DsRed1. Optical filters were supplied by BD Biosciences or purchased from Omega Optical, Inc. or Chroma Technology, Corp. (Figure 3A). Spectral overlap of the proteins in flow cytometry can be dealt with by employing the appropriate optical filter configuration as well as real-time electronic (hardware) or post-acquisition software-based compensation (3).

At 458 nm, all of the fluorescent proteins were excited. While both EGFP and ECFP excited at 60 percent of the maximum absorbance, DsRed1 excited at less than 20 percent of the maximum absorbance, and EYFP excited at less than 10 percent of the maximum absorbance. We achieved resolution of EGFP, ECFP, and EYFP by intra-laser compensation (Figure 3A). Use of a 610/30 BP filter removed any interference of signals from the GFP variants and in the DsRed1 fluorescence channel. Since DsRed1 has a red-shifted spectrum, fluorescence generated at either 458-nm or 568-nm excitation did not interfere with the signals of the GFP variants. Furthermore, no signals were elicited from the GFP variants at 568 nm. Using these conditions, we successfully resolved all four proteins in real time by careful pair-wise compensations between detectors (Figure 3B).

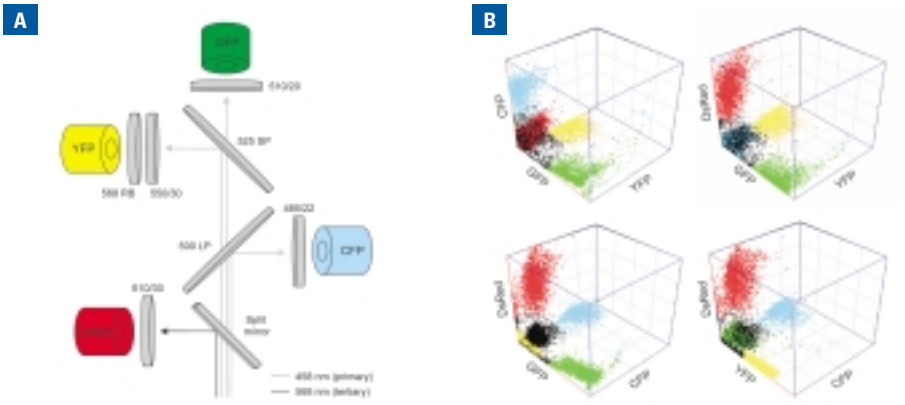

Figure 3. Real-time flow cytometric analysis of four fluorescent proteins. Panel A. Optical configuration used for detection of DsRed1, EYFP, EGFP, and ECFP using dual-laser excitation at 458 and 568 nm. Cells were analyzed on a FACSVantage SE (BD Biosciences Immunocytometry Systems) equipped with Coherent I-90 argon-ion and Coherent I-302C krypton-ion lasers (Coherent Inc). The primary argon-ion laser was tuned to 458 nm (100 mW) and the krypton-ion laser was tuned to 568 nm (35 mW). The three-beam separation option allowed the krypton-ion laser beam to be directed along the third laser pathway (tertiary position), maximizing spatial separation of the two beams, and minimizing both laser noise and crosstalk between the signals. Data were acquired using CELLQuest. The OmniCompensation option allowed real-time intra- and inter-laser compensations. The ECFP signal was split off from the EYFP/EGFP signals using a 500 nm longpass (LP) dichroic filter and collected with a 485/22 nm bandpass filter. The EYFP and EGFP signals were separated with a 525 nm shortpass (SP) dichroic filter and collected with a 550/30 nm bandpass filter and a 510/20 nm bandpass filter, respectively. A 568 nm restriction band (RB) filter was placed in front of the 550/30 nm bandpass filter to block off stray laser light. The DsRed1 signal was collected with a 610/30 nm bandpass filter with a sharp cutoff at 595 nm. **Panel B.** Three-dimensional plots showing real-time detection of DsRed1, EYFP, EGFP, and ECFP signals in a mixture of GP+E-86 cells individually expressing the MRIN, MYIN, MGIN, and MCIN retroviral vectors (together with nontransduced parental GP+E-86 cells). Plots were generated using WinMDI v2.7 (from J. Trotter, The Scripps Research Institute, La Jolla, CA/BD Biosciences Pharmingen). Reproduced from Hawley *et al.* (3) by permission of AlphaMed Press.

Since many flow cytometers are equipped with only an argon-ion laser tunable to 488 nm, we analyzed the combination of DsRed1/EYFP/EGFP-expressing cell population using single-laser excitation at 488 nm. DsRed1 has a minor excitation peak around 488 nm and can be excited reasonably well (40 percent of maximum absorbance) at this wavelength. These three proteins were readily detectable and distinguishable in real time (data not shown; 2).

Sort subpopulations of viable cells

Through retrovirally mediated gene transfer of DsRed1, EYFP, EGFP, and ECFP-containing vectors, we established permanent mammalian cell lines expressing these proteins. We demonstrated that it is possible to differentiate among DsRed1, EYFP, EGFP, and ECFP using dual-laser flow cytometry, making this a useful technique to sort subpopulations of viable cells simultaneously expressing DsRed1 and various combinations of the three GFP variants.

Product	Size	Cat. #
pDsRed1-1 Vector	20 µg	6922-1
pEGFP-1 Vector	20 µg	6086-1
pEYFP-N1 Vector	20 µg	6006-1
pECFP Vector	20 µg	6075-1
MSCV Retroviral Expression System	each	K1062-1

References

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